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A bovine model of rhizomelic chondrodysplasia punctata caused by a deep intronic splicing mutation in the GNPAT gene

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1 **A bovine model of rhizomelic chondrodysplasia punctata**
2 **caused by a deep intronic splicing mutation in the *GNPAT***
3 **gene**

4

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38 **Abstract**

39 **Background**

40 Genetic defects that occur naturally in livestock species provide valuable models for
41 investigating the molecular mechanisms underlying rare human diseases. Livestock breeds are
42 subject to the regular emergence of recessive genetic defects, due to their low genetic
43 variability, while their large population sizes provide easy access to case and control
44 individuals, as well as massive amounts of pedigree, genomic and phenotypic information
45 recorded for selection purposes. In this study, we investigated a lethal form of recessive
46 chondrodysplasia observed in 21 stillborn calves of the Aubrac breed of beef cattle.

47 **Results**

48 Detailed clinical examinations revealed proximal limb shortening, epiphyseal calcific deposits
49 and other clinical signs consistent with human rhizomelic chondrodysplasia punctata, a rare
50 peroxisomal disorder caused by recessive mutations in one of five genes (*AGPS*, *FARI*,
51 *GNPAT*, *PEX5* and *PEX7*). Using homozygosity mapping, whole genome sequencing of two
52 affected individuals, and filtering for variants found in 1,867 control genomes, we reduced the
53 list of candidate variants to a single deep intronic substitution in *GNPAT* (g.4,039,268G>A on
54 Chromosome 28 of the ARS-UCD1.2 bovine genome assembly). For verification, we
55 performed large-scale genotyping of this variant using a custom SNP array and found a
56 perfect genotype-phenotype correlation in 21 cases and 26 of their parents, and a complete
57 absence of homozygotes in 1,195 Aubrac controls. The g.4,039,268A allele segregated at a
58 frequency of 2.6% in this population and was absent in 375,535 additional individuals from
59 17 breeds. Then, using *in vivo* and *in vitro* analyses, we demonstrated that the derived allele
60 activates cryptic splice sites within intron 11 resulting in abnormal transcripts. Finally, by

61 mining the wealth of records available in the French bovine database, we demonstrated that
62 this deep intronic substitution was responsible not only for stillbirth but also for juvenile
63 mortality in homozygotes and had a moderate but significant negative effect on muscle
64 development in heterozygotes.

65 **Conclusions**

66 We report the first spontaneous large animal model of rhizomelic chondrodysplasia punctata
67 and provide both a diagnostic test to counter-select this defect in cattle and interesting insights
68 into the molecular consequences of complete or partial GNPAT insufficiency in mammals.

69

70 **Background**

71 Over the last decade, the advent of high-throughput genotyping and next-generation
72 sequencing have dramatically advanced clinical research, leading to the identification of
73 thousands of disease-causing variants in humans and non-model species [1]. However, most
74 of genetic studies are biased by a tendency to focus on the exome because of the challenges of
75 annotating non-coding regions of the genome and, to a lesser extent, to the advantages of
76 whole-exome sequencing over whole-genome sequencing [2]. While increasing evidence
77 points to the role of non-coding variations in the onset of diseases [3], their study in humans
78 suffers from several limitations and the pathophysiology of many disorders remains
79 unresolved. The main limiting factors include the small number of patients affected by rare
80 genetic defects and the relatively high genetic variability of our species, which makes it
81 difficult to filter variants in the absence of functional annotation. In addition, the difficulty in
82 obtaining a variety of tissues from living or deceased patients due to health risks, ethical or
83 religious concerns often hinders in-depth clinical investigation and functional validation. In
84 this context, naturally occurring genetic defects in livestock species represent valuable models
85 to study the molecular mechanisms underlying rare human diseases. Indeed, farm animals are
86 divided into numerous inbred populations or breeds that are prone to the regular emergence of
87 recessive genetic defects [4]. In addition, their large population sizes provide easy access to
88 case and control individuals, as well as massive amounts of pedigree, genomic and phenotypic
89 information recorded for selection purposes [5,6].

90 From 2002 to 2020, 21 stillborn Aubrac calves with severe skeletal dysplasia were reported to
91 the French National Observatory for Bovine Abnormalities (ONAB) for initial suspicion of
92 Bulldog Calf Syndrome (BDS), a congenital form of bovine chondrodysplasia previously
93 described to be associated with mutations in the aggrecan (*ACAN*), and collagen type II alpha

94 1 chain (COL2A1) genes [5,7–13]. Pathological examination and pedigree analysis revealed
95 that this new genetic defect was actually similar to human rhizomelic chondrodysplasia
96 punctata (RCDP) [14]. RCDP is a recessive peroxisomal disease caused by mutations in five
97 genes: *AGPS*, *FARI*, *GNPAT*, *PEX5* and *PEX7* encoding the alkylglycerone phosphate
98 synthase, the fatty alcohol reductase 1, the glyceronephosphate O-acyltransferase, and the
99 peroxisomal biogenesis factor 5 and 7, respectively [15–20].

100 In this article we describe, how we were able to reduce the list of candidate causative variants
101 to a single deep intronic substitution in *GNPAT*, and to characterize its molecular and clinical
102 consequences in the homozygous and heterozygous states, by exploiting the wealth of
103 resources available in cattle. In other words, we report the first large animal model of RCDP
104 and an original example of a deep intronic mutation responsible for a genetic defect in
105 livestock.

106

107 **Methods**

108 **Animals**

109 Twenty-one stillborn calves (8 males and 13 females) affected by a severe form of skeletal
110 dysplasia were observed in 21 purebred Aubrac herds over an 18-year period. Seventeen of
111 the breeders kept genealogical records, which were extracted from the French national
112 pedigree database. Veterinarians and artificial insemination (AI) technicians performed a
113 gross clinical description in the field and collected ear biopsies and photographs for all
114 affected calves. Due to the rapid removal of carcasses by rendering companies, only three
115 affected calves could be recovered for full necropsy and pathological examination. The body
116 of an unaffected calf that died of natural causes at four days of age was also collected to serve

117 as a control. At the time of the study, biological samples for DNA extraction were also
118 available for 15 dams and 11 sires of the cases and for one of their common ancestors, the AI
119 bull "E.". In addition, after identification of the *GNPAT* candidate causal variant, blood from
120 three heterozygous mutant and three wild-type cows was collected in PAXgene Blood RNA
121 Tubes (Qiagen) to perform RNA extractions and RT-PCR analysis. Finally, whole genome
122 sequences, SNP array genotypes, and phenotypes from thousands to hundreds of thousands of
123 animals from numerous breeds collected in the framework of other projects were also used in
124 this study. Details of this additional material and on the analyses performed on it are given
125 below.

126

127 **Pedigree analysis**

128 Genealogical information was extracted from the French national pedigree database for 17 of
129 the affected calves and 110,247 control calves born between 2019 and 2021 with both parents
130 recorded. A search for common ancestry between the parents of the affected calves was
131 performed using the `anc_comm` option of the `pedig` package [21]. In parallel, the genetic
132 contribution to the case and control populations was estimated for each ancestor using the
133 `prob_orig` option of the same package. Then, for individuals with a genetic contribution
134 greater than or equal to 1% in each population, the ratio "contribution to the case
135 population/contribution to the control population" was calculated.

136

137 **Necropsy and pathological examination**

138 The frozen bodies of one control and three affected calves were subjected to digital
139 radiography (XDR1, Canon Medical Systems), computed tomography (CT) scanning (80-

140 slice CT scanner, Aquilion lighting, Canon Medical Systems) and necropsy at the National
141 Veterinary School of Alfort. In addition to the classic post-mortem examination, special
142 attention was given to the deformities of the skull and limb bones. The heads were sawed
143 through the midline, and the left limbs were harvested, boiled, cleaned of residual soft tissue,
144 and bleached with 5% hydrogen peroxide, prior to partial skeletal reconstruction.

145

146 **DNA extraction**

147 Genomic DNA was extracted from blood using the Wizard Genomic DNA Purification Kit
148 (Promega) and from ear biopsies or semen using the Genra Puregene Cell and Tissue Kit
149 (Qiagen). DNA purity and concentration were evaluated using a NanoDrop spectrophotometer
150 (ThermoFisher Scientific).

151

152 **Homozygosity mapping**

153 The twenty-one Aubrac cases, 26 of their parents and 1548 control animals from the same
154 breed were genotyped with various Illumina SNP arrays over time (Bovine SNP50,
155 EuroG10K and EuroGMD). Genotypes were phased and imputed to the Bovine SNP50 using
156 FImpute3 [22] in the framework of the French genomic evaluation, as described in Mesbah-
157 Uddin *et al.* [23]. The position of the markers was based on the ARS-UCD1.2 bovine genome
158 assembly. We then considered sliding haplotypes of 20 markers (~1 Mb) and we computed
159 Fisher's exact tests on 2x2 contingency tables consisting of the number of homozygous and
160 “non-homozygous” animals in the case and control groups. A Bonferroni correction was
161 applied to account for multiple testing (n=78861 tests with at least one homozygous carrier in
162 the case group) and therefore the 0.05 significance threshold was set at $-\log P=6.20$.

163

164 **Analysis of Whole Genome Sequences**

165 The genomes of two RCDP-affected calves were sequenced at 19.2x and 19.4x coverage on
166 an Illumina NovaSeq6000 platform in 150 paired-end mode, after library preparation using
167 the NEXTFlex PCR-Free DNA Sequencing Kit (Perkin Elmer Applied Genomics). Reads
168 were aligned to the ARS-UCD1.2 bovine genome assembly [24] with the Burrows–Wheeler
169 aligner (BWA-v0.6.1-r104; [25]) prior to the identification of SNPs and small InDels using
170 the GATK-HaplotypeCaller software [26] as previously described in Daetwyler *et al.* and
171 Boussaha *et al.* [26, 27]. Putative structural variations (SVs) were detected using the Pindel
172 [28], Delly [29], and Lumpy [30] software and recorded if they were scored by at least two
173 tools in the same individual. These variants were compared with those found in 1,867 control
174 genomes in a previous study using the same procedure [6]. The control genomes included 39
175 Aubrac individuals, all of whom were non-carriers of the 35-marker haplotype common to
176 affected calves based on phased and imputed Illumina BovineSNP50 array genotypes, and
177 representatives of more than 70 cattle breeds or populations (Additional file 1: Table S1).
178 Only SNPs, InDels and SVs located within the mapping interval (Chr28:3,555,723-5,143,700
179 bp), observed in the homozygous state in both cases and absent in all controls were
180 considered as candidate variants. The only remaining variant after filtering, in this case
181 variant g.4,039,268G>A on Chr28 were annotated using Variant Effect Predictor (Ensembl
182 release 110; <https://www.ensembl.org/info/docs/tools/vep/index.html>) [31].

183

184 **Validation of the variant g.4,039,268G>A by Sanger sequencing and large-** 185 **scale genotyping**

186 As a first verification, we genotyped the variant g.4,039,268G>A using PCR and Sanger
187 sequencing in 6 Aubrac cattle (2 affected calves, 2 heterozygous parents of cases, and two
188 non-carriers based on haplotype information). A segment of 640 bp was amplified in a
189 Mastercycler pro thermocycler (Eppendorf) using primers 5'-
190 TCCCTTCCTTCAAGGCTACA-3' and 5'-GTTAGGAGCCAGAGCAGCAC-3' and the Go-
191 Taq Flexi DNA Polymerase (Promega), according to the manufacturer's instructions.
192 Amplicons were purified and bidirectionally sequenced by Eurofins MWG (Hilden, Germany)
193 using conventional Sanger sequencing. Electropherograms were analyzed using NovoSNP
194 software for variant detection [32].

195 In addition, to genotype this variant on a large scale, we added a probe to the Illumina
196 EuroGMD SNP array using the following design:
197 TTTGTTTCAGTAGGAAGTGAGGGCAGCCATTTTGAGCATAACATGATTCTCAGTGT
198 TTTTC[A/G]NNCTTGCCGCATGCACTTTTGTTTAAATGTGAGGAGAGTATGGCTGT
199 ATACAAAGTGAAA. The EuroGMD SNP array is routinely used for genomic evaluation in
200 France and genotypes of 21 affected calves and 376,730 controls from 19 French breeds
201 (including 1,195 Aubrac cattle) were available at the time of writing.

202

203 **Minigenes construction**

204 A 1149 bp fragment containing exon 11, intron 11 and exon 12 of the *GNPAT* gene was
205 amplified from the genomic DNA of a homozygous carrier of the Chr28 g.4,039,268A mutant
206 allele. BamHI and XhoI restriction sites were incorporated into primers 5'-
207 TACCGAGCTCGGATCCTCCAGAGGATGTCTACAGTTGC-3' and 5'-
208 GCCCTCTAGACTCGAGTTGCAAAGATTTACACACCTGA-3' designed for this purpose.
209 PCR was performed in a 25 µl reaction mixture containing 12.5µL 2X KAPA HiFi HotStart

210 ReadyMix (Roche), 50 ng genomic DNA, and 0.3 μ M each primer. The PCR program
211 comprised an initial denaturation at 95 °C for 3 min followed by 30 cycles of denaturation at
212 98 °C for 20 s, annealing at 65 °C for 15 s, extension at 72 °C for 1 min, and a final extension
213 at 72 °C for 1 min 20 s. The PCR products were cloned into the pcDNA3.1(+) vector
214 (Invitrogen) that was linearized by restriction enzymes BamHI and XhoI, using the T4 DNA
215 Ligase (New England Biolabs) in accordance with the manufacturer's instructions. The
216 resulting minigene construct carrying the alternative g.4,039,268A allele was termed
217 pcDNA3.1-GNPAT_A. The g.4,039,268G reference allele was then introduced into the
218 pcDNA3.1-GNPAT_A minigene construct by site directed mutagenesis to obtain the
219 pcDNA3.1-GNPAT_G minigene construct. This was achieved by means of the QuikChange
220 II XL Site-Directed Mutagenesis Kit (Agilent) using primers 5'-
221 CTCAGTGTTTTTCGGACTTGCCGCATGC-3' and 5'-
222 GCATGCGGCAAGTCCGAAAAACACTGAG-3' in accordance with the manufacturer's
223 instructions. Sequences of both minigenes were verified by Sanger sequencing using T7 and
224 BGH universal primers in addition to primer 5'-CAAGTGGGTCTGGGGTCTG-3'.

225

226 **Cell culture and transfection**

227 Human embryonic kidney (HEK) 293T cells were maintained in DMEM supplemented with
228 10% fetal calf serum (Gibco). Cells were seeded with 300 000 cells/well in 6-well plates and
229 transfected 24 hours later with 1 μ g of each minigene construct mixed with 3 μ L of
230 Lipofectamine 2000 Reagent (Invitrogen) per well, according to the manufacturer's
231 instructions. Four hours after transfection, media were replaced by DMEM supplemented with
232 10% fetal calf serum and maintained in an incubator at 37°C and 5% CO₂. Forty-eight hours

233 after transfection, the cells were washed with phosphate-buffered saline (PBS) and lysed with
234 RLT buffer (Qiagen).

235

236 **RNA extraction from cell culture and RT-PCR analysis**

237 Total RNA was extracted from lysed transfected cells by means of the RNeasy Mini Kit
238 according to the manufacturer's instructions (Qiagen). The RT step was performed using the
239 SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen) with 400 ng RNA,
240 OligodT20 5µM, 500 µM each dNTP, MgCl₂ 5 mM, DTT 0.01M, 40 U RNaseOUT and 200
241 U Superscript 3 following the manufacturer's instruction. RT products were treated with 2U
242 RNase H during 20 min at 37°C. The PCR step was achieved using primers forward T7 (5'-
243 TAATACGACTCACTATAGGG-3') and reverse BGH (5'-TAGAAGGCACAGTCGAGG-
244 3') located within the 5'- and 3'-untranslated regions of pcDNA3.1-GNPAT minigene
245 constructs, respectively. The reaction was performed in a 50-µL mixture containing 1.25 U
246 GoTaq DNA polymerase (Promega), 200 µM dNTPs, 2 µL cDNA and 0.5 µM of each primer.
247 The PCR program had an initial denaturation at 95°C for 2 min, followed by 30 cycles of
248 denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min 30 s,
249 and a final extension step at 72°C for 5 min.

250

251 **RNA extraction from blood and RT-PCR analysis**

252 RNA was extracted from blood samples collected in PAXgene Blood RNA Tubes using the
253 PAXgene Blood RNA Kit (Qiagen). Tubes were stored at -20°C for one month and thawed at
254 room temperature for 2 hours before RNA extraction. Next, they were centrifuged 10 min at
255 4000 g and the supernatant was gently discarded by pouring off the tube. RNA was extracted
256 from cell pellets by following the manufacturer's guidelines, and their purity and integrity

257 were assessed with the Bioanalyzer 2100 (Agilent). A 1 µg commercial sample of bovine
258 muscle RNA (Gentaur) was used as a positive control. The reverse transcription (RT) step
259 was performed using the SuperScript III First-Strand Synthesis System for RT-PCR
260 (Invitrogen) with 60 ng RNA, OligodT20 5µM, 500 µM each dNTP, MgCl₂ 5 mM, DTT
261 0.01M, 40U RNaseOUT and 200 U Superscript 3 following the manufacturer's instruction.
262 RT products were treated with 2U RNase H for 20 min at 37°C. The PCR step was achieved
263 using primers 5'-GCTTTCGCTTCCTATGCAGT-3' and 5'-
264 TGTC CCTCGTCATCACTTGT-3' located within the *GNPAT* exon 11 and 12, respectively.
265 Each cDNA sample was amplified in quadruplicate in a 25-µL mixture containing 0.75 U
266 GoTaq DNA polymerase (Promega), 200 µM dNTPs, 1 µL cDNA and 0.5 µM of each primer.
267 The PCR program had an initial denaturation at 95°C for 2 min, followed by 45 cycles of
268 denaturation at 95°C for 30 s, annealing at 53°C for 30 s, extension at 72°C for 1 min, and a
269 final extension step at 72°C for 10 min. The four PCR replicates obtained from each sample
270 were pooled and concentrated with the MinElute PCR Purification Kit (Qiagen) before gel
271 electrophoresis.

272

273 **Prediction of exonic splicing enhancers (ESE) and protein structure analysis**

274 ESE motif prediction was performed using ESEfinder 3.0 software in the context of the A and
275 G alleles to identify the creation or disruption of putative splicing regulatory elements
276 (<http://krainer01.cshl.edu/cgi-bin/tools/ESE3/ese finder.cgi?process=home>; [33,34]). The
277 coding sequence of normal and abnormal GNPAT transcripts was translated into amino acid
278 sequences using ExpASy (<https://web.expasy.org/translate/>; [35]). Information about protein
279 domains was obtained from UniProt (<https://www.uniprot.org/uniprotkb/A4IF87/entry>,
280 accessed 08.05.2020) and from Ofman *et al.* [18].

281

282 **Effects of allele Chr28 g.4,039,268A on juvenile mortality rates**

283 The phenotypic effects of four mating types on juvenile mortality rates were evaluated using
284 records from the French national bovine database and the following fixed-effect model:

$$y_{ij} = \mu + m_j + e_{ij}$$

285 where y_{ij} represents the phenotype of interest, μ is the overall phenotypic mean, m_j is the
286 fixed effect of the mating status, and e_{ij} is the random residual error. The analysis was
287 performed using the GLM procedure of SAS software (version 9.4; SAS Institute Inc., Cary,
288 NC). The mating types considered were named 1 x 1, 1 x 0, 0 x 1, and 0 x 0, where the first
289 position corresponds to the genotype of the sire and the second position corresponds to the
290 genotype of the maternal grandsire in terms of allele dosage for the g.4,039,268A allele.
291 Juvenile mortality was examined during four periods commonly considered in the literature
292 (0-2, 3-14, 15-55 and 56-365 days after birth; e.g. [36,37]) as well as for a combination of the
293 first two periods. For each time window, the mortality rates were calculated by dividing the
294 number of calves that died of natural causes during the period by the number of calves present
295 at the beginning of the period. We also calculated the expected effect on juvenile mortality
296 rates, assuming a full penetrance of lethality under homozygosity, using the formula
297 $\frac{1}{4(2-fa)}(1 - \mu)$ adapted from Fritz *et al.* [38], where fa is the population frequency of the
298 g.4,039,268A allele, and μ is the phenotypic mean of the trait.

299

300 **Effects of heterozygosity for the Chr28 g.4,039,268A allele on performance** 301 **traits**

302 The Aubrac is one of the 9 breeds included in the French national genetic evaluation of beef
303 cattle. Animals are genetically evaluated each year using the national polygenic BLUP
304 evaluation for five traits measured in the commercial farms: birth weight, ease of calving,
305 muscular development, skeletal development and weight at 210 days. Genetic breeding values
306 and residuals were extracted from the French national database for genotyped animals and
307 summed to obtain a phenotype adjusted for non-genetic effects. The effect of the
308 g.4,039,268A allele was tested using the GWAS method for the five traits studied with the
309 GCTA software version 1.26 [39], using the mlma option, and applied to the following mixed
310 linear model:

$$\mathbf{y} = \mathbf{1}\mu + \mathbf{x}\mathbf{b} + \mathbf{u} + \mathbf{e}$$

311 where \mathbf{y} is the vector of corrected phenotypes; μ is the overall mean; $\mathbf{1}$ is a vector of ones; \mathbf{b} is
312 the additive effect of the derived allele; \mathbf{x} is the genotype for the SNP; $\mathbf{u} \sim N(\mathbf{0}, \mathbf{G} \sigma_u^2)$ is the
313 vector of random polygenic effect, where \mathbf{G} is the genomic relationship matrix calculated
314 using the 50K SNP genotypes (computed without Chr 28), and σ_u^2 is the polygenic variance
315 that is estimated based on the null model without the SNP effect; and $\mathbf{e} \sim N(\mathbf{0}, \mathbf{I} \sigma_e^2)$ is the
316 vector of random residual effects, where \mathbf{I} the identity matrix and σ_e^2 the residual variance.
317 The number of animals analyzed ranged from 4,401 to 8,416 depending on the trait, of which
318 44% had a status based on direct genotyping of the variant and 56% based on a haplotype test
319 considering the 35-marker haplotype (from positions 3,583,342 bp to 5,092,017 bp on the
320 bovine reference genome assembly ARS-UCD1.2 [24]) identified by homozygosity mapping
321 (see Results).

322

323 **Results**

324 **Pedigree analysis suggests an autosomal recessive mode of inheritance**

325 The cases consisted of eight males and thirteen females born to unaffected parents over an 18-
326 year period in 21 purebred Aubrac herds spread throughout France. Analysis of the pedigrees
327 of 17 cases with information available back to the 1960's revealed several recent inbreeding
328 loops supporting an autosomal recessive mode of inheritance, but did not allow us to identify
329 a single ancestor shared by all their parents. Further analysis highlighted the bull "E." (born in
330 1989) as the most influential spreader of this putative recessive defect in recent decades, with
331 a ratio of 2.04 between its genetic contributions to the case group (3.20 %) and to 110,247
332 controls born between 2019 and 2021 (1.57%; Fig. 1 a). This AI bull was present in the
333 genealogy of 12/17 cases (Fig. 1 b).

334

335 **Clinical findings are compatible with RCDP**

336 The 21 affected calves were stillborn and exhibited extremely disproportionate dwarfism
337 characterized by craniofacial dysmorphism, short limbs with hypermobile joints, a distended
338 abdomen prone to eventration, and low birth weight despite normal gestation length (i.e., ~20-
339 30 kg versus ~40 kg; Fig. 2). Due to the rapid collection of dead animals by rendering
340 companies, only three affected calves (two females, one male) were available for extensive
341 pathological examination. Radiographs, CT scans, and longitudinal skull sections allowed
342 better characterization of the craniofacial dysmorphism. The latter consisted primarily of
343 severe hypoplasia of the maxilla and secondary deformities of neighbouring bones and soft
344 tissue structures, resulting in a cleft palate, curvature of the mandible, protrusion of the
345 tongue, bossing of the frontal bone, and the presence of an anterior fontanelle (Fig. 2).
346 Imaging and skeletal preparation also revealed platyspondyly of the thoracic and lumbar
347 vertebrae, abnormally short ribs, and rhizomelic limb shortening (Additional file 2: Figure S1;

348 Fig. 3). More specifically, the proximal long bones had shortened diaphyses and enlarged
349 metaphyses with thickened cortex, whereas the diaphyses of the distal long bones (metatarsus,
350 metacarpus, and phalanges) were normally developed. In addition, the tuberosity of the
351 calcaneus, the femoral head and all epiphyses were absent or reduced to punctate
352 calcifications (Fig. 3). Finally, the necropsy revealed hyperlaxity of all joints except the stifle
353 and hock, which were affected by arthrogryposis, and no particular malformations of the
354 internal organs.

355 Based on all of these elements, we arrived at the diagnosis of rhizomelic chondrodysplasia
356 punctata (RCDP).

357

358 **Mapping and identification of a candidate causal variant in *GNPAT***

359 As a first step to gain insight into the molecular etiology of this bovine form of RCDP, we
360 used a homozygosity mapping approach. By analyzing Illumina BovineSNP50 genotypes of
361 21 case and 1628 control animals for sliding windows of 20 markers, we mapped the RCDP
362 locus at the beginning of chromosome 28 (Fig. 4 a). Under the peak position, we identified a
363 35-marker haplotype (from positions 3,583,342 bp to 5,092,017 bp on the bovine reference
364 genome assembly ARS-UCD1.2) that was observed in the homozygous state in all the
365 affected animals and in none of the controls. The most proximal markers outside of this
366 segment defined the borders of a 1.6 Mb mapping interval (Chr28:3,555,723-5,143,700 bp)
367 containing *GNPAT* and 11 additional genes (*Homo C1orf198*, *TTC13*, *ARV1*, *FAM89A*,
368 *TRIM67*, *Homo C1orf131*, *EXOC8*, *SPRTN*, *EGLN1*, *TNSAX*, *DISC1*; Fig. 4 b).

369 Next, we sequenced the complete genomes of two RCDP-affected calves and compared them
370 with 1,867 control genomes (Additional file 1: Table S1). The latter consisted of 39 non-
371 carrier Aubrac individuals (based on haplotype information) as well as representatives of

372 more than 70 breeds. Within the mapping interval, we identified a total of 3,115 sequence
373 variants for which both cases were homozygous for the alternative allele (Additional file 3:
374 Table S2). Subsequent filtering for variants completely absent in the controls reduced the list
375 to a single candidate: a deep intronic substitution located 549 bp downstream and 323 bp
376 upstream of *GNPAT* exons 11 and 12, respectively (Chr28 g.4,039,268G>A; Fig. 4 c, d).
377

378 **Validation of the *GNPAT* g.4,039,268G>A variant by large-scale genotyping**

379 As a first verification, we genotyped the *GNPAT* g.4,039,268G>A variant in the 21 cases, all
380 their available parents (n=26), and the AI bull “E.” using the Illumina EuroGMD custom SNP
381 array. As expected, each case was homozygous for the derived allele while all unaffected
382 parents and “E.” were heterozygous. For further validation, we extended the analysis to 1,195
383 unaffected Aubrac cattle and 375,535 controls from 17 breeds genotyped on the same array
384 for genomic evaluation purposes. The g.4,039,268A allele was found to segregate only in
385 Aubrac cattle at a frequency of 2.60% and we did not observe any homozygous carriers
386 (Table 1).

387

Breeds	Genotypes			f(A)
	GG	AG	AA	
Abondance	5,789	-	-	0
Aubrac	1,136	59	-	2.60
Blonde d'Aquitaine	6,114	-	-	0
Bretonne pie noir	43	-	-	0
Brown swiss	4,648	-	-	0
Charolaise	11,896	-	-	0
Créole	91	-	-	0
Holstein	243,189	-	-	0
Jersey	2,991	-	-	0
Limousine	2,391	-	-	0
Montbéliarde	141,296	-	-	0
Normande	31,745	-	-	0
Parthenaise	1,005	-	-	0

Rouge des prés	17	-	-	0
Salers	1,007	-	-	0
Simmental	4,914	-	-	0
Tarentaise	2,994	-	-	0
Vosgienne	758	-	-	0

388 **Table 1. Results of large-scale genotyping of the *GNPAT* g.4,039,268G>A variant in**
389 **376,730 unaffected animals from 18 breeds.** The number of animals is given for each
390 genotype per breed; f(A): frequency of the g.4,039,268A allele.

391

392 **Chr28 g.4,039,268A allele activates cryptic splice sites in *GNPAT* intron 11**

393 Following these preliminary verifications, we performed a series of analyses to investigate the
394 effects of the g.4,039,268A variant on *GNPAT* function.

395 Because tissues from RCDP-affected calves were collected and frozen at -20°C only prior to
396 the discovery of the candidate variant, they were not available for a posteriori RNA
397 extraction. Therefore, we attempted to perform a Western blot analysis using an antibody
398 directed against the N-terminal region of the *GNPAT* protein (ab75060, Abcam).

399 Unfortunately, we were unable to detect a signal of the expected molecular weight in wild-type
400 samples, either with proteins extracted in our laboratory from the muscle of a control animal
401 or with a commercial extract (BT-102, GENTAUR; results not shown). Although this
402 antibody has been used successfully against both human and mouse *GNPAT* [40,41], we
403 concluded that it does not work with the bovine orthologous protein.

404 After this unsuccessful attempt, we performed a minigene analysis to investigate *in vitro* a
405 possible effect of the Chr28 g.4,039,268A allele on altering *GNPAT* splicing. We constructed
406 two expression plasmids containing exon 11, intron 11 and exon 12 of the *GNPAT* gene and
407 either the ancestral or the derived allele of the deep intronic variant (pcDNA3.1-*GNPAT*_G
408 and pcDNA3.1-*GNPAT*_A, respectively; Fig. 5 a). RT-PCR analysis of HEK293T cells
409 transfected with both minigenes showed only one major specific transcript for each

410 construction, but of different sizes (Fig. 5 a). Sanger sequencing of the amplicons revealed
411 that transcript No. 1 from pcDNA3.1-GNPAT_G was fully spliced and corresponded to exon
412 11/exon 12 whereas transcript No. 2 from pcDNA3.1-GNPAT_A corresponded to exon 11
413 and exon 12 separated by a small portion of intron 11 consisting of an 86 bp cryptic exon
414 (Chr28:4,039,260-4,039,345; Fig. 5 b, c). Consistent with this observation, sequence analysis
415 using the ESEfinder 3.0 software revealed that allele A was predicted to increase the binding
416 capacity of the SF2/ASF splicing factor at the 5' end of the cryptic exon, which may explain
417 the selective inclusion of the latter in transcript No. 2 (Fig. 5 c). As a final step, we performed
418 RT-PCR analyses on total blood RNA extracted from three heterozygous (HT) and three wild-
419 type (WT) Aubrac cattle to verify the effects of the deep intronic variant *in vivo*. cDNA
420 amplification with primers targeting *GNPAT* exons 11 and 12, followed by agarose gel
421 electrophoresis, yielded four distinct bands that were purified and sequenced by Sanger's
422 method (Fig. 5 d). Bands 1 and 4 were observed in both WT and HT animals and
423 corresponded to amplicons with exon 11 and 12, and either fully spliced or unspliced intron
424 11, respectively. In contrast, bands 2 and 3 were observed exclusively in HT animals and
425 resulted from abnormal splicing of intron 11. Between exons 11 and 12, PCR product 2
426 contained the same 86 bp cryptic exon observed in minigene analysis, whereas PCR product 3
427 also contained the portion of the intron 11 located between exon 11 and the cryptic exon, in
428 addition to the latter.

429 Taken together, the results of our *in vitro*, *in silico* and *in vivo* analyses support that the Chr28
430 g.4039268A allele alters the *GNPAT* splicing by activating cryptic splice sites within intron
431 11. Incorporation of all or part of the latter intron into the *GNPAT* mRNA is predicted to
432 cause frameshifts and to generate mutant proteins lacking the last 21% amino acids of the
433 bovine GNPAT protein and, in particular, the C-terminal microbody targeting signal (Fig. 5
434 e).

435

436 **Mining the large dataset of records from the French national bovine database**
437 **to study the effects of the Chr8 g.4,039,268A allele**

438 As a final step to complete our study, we mined the large dataset of records from the French
439 national bovine database to gain further insight into the phenotypic consequences of the
440 Chr28 g.4,039,268A allele in the heterozygous or homozygous states.

441 First, we investigated the penetrance and expressivity of this allele by examining four juvenile
442 mortality rates for different types of matings between genotyped sires and daughters of
443 genotyped sires using a fixed effect model (Table 2). We observed a significant increase in
444 mortality rates in at-risk (i.e., where the sire and maternal grandsire are both heterozygous; 1
445 x 1) versus control (wild-type sire and maternal grandsire; 0 x 0) matings for the 0-2 day
446 period (+5.69 %; p-value <0.0001) and also for the 3-14 day period (+1.28%; p-value=0.02),
447 indicating that a fraction of homozygous mutant calves were not stillborn but died a few days
448 later. Combining these periods, the increase in mortality reached +6.89% within the first 2
449 weeks after birth (p-value <0.0001), which is only about half of the +12.30% increase in
450 mortality expected in at-risk matings assuming complete penetrance (see Methods for
451 calculation details). To assess whether this difference between the expected and observed
452 increase in the mortality rate was due to incomplete penetrance or to underreporting of
453 stillbirths, we examined the 21 cases reported to the ONAB and found that only 61.90%
454 (13/21) of them had been ear-tagged and officially registered in the French national bovine
455 database. Considering that these two proportions (observed/expected increase in mortality
456 within the first 2 weeks= +6.89 %/+12.30%=56.02% and ear-tagged individuals/cases
457 reported=61.90%) were not significantly different among 21 individuals using a Chi2
458 goodness of fit test (12:9 vs 13:8; p=1), we concluded that the penetrance of peri- and

459 postnatal mortality is most likely complete in homozygous carriers of the Chr8 g.4,039,268A
 460 allele.

461

Trait	Mating	Nb	Raw mean (%)	Difference (%)	SE (%)	P-value
56-365 days mortality rate	1 x 1	268	1.49	0.47	0.62	0.44
	1 x 0	4078	1.08	0.06	0.16	0.72
	0 x 1	4973	0.95	-0.08	0.15	0.60
	0 x 0	91703	1.02	-	-	-
15-55 days mortality rate	1 x 1	271	1.11	0.37	0.53	0.48
	1 x 0	4,111	0.80	0.07	0.14	0.63
	0 x 1	5,022	0.94	0.20	0.13	0.11
	0 x 0	92,397	0.74	-	-	-
3-14 days mortality rate	1 x 1	277	2.17	1.28	0.57	0.02
	1 x 0	4,144	0.80	-0.09	0.15	0.54
	0 x 1	5,077	1.08	0.20	0.14	0.15
	0 x 0	93,227	0.89	-	-	-
0-2 days mortality rate	1 x 1	300	7.67	5.69	0.81	1.59*10 ⁻¹¹
	1 x 0	4,248	2.45	0.47	0.22	0.03
	0 x 1	5,178	1.95	-0.03	0.20	0.89
	0 x 0	95,109	1.98	-	-	-
0-14 days mortality rate	1 x 1	300	9.67	6.82	0.97	1.32*10⁻¹¹
	1 x 0	4,248	3.23	0.38	0.26	0.15
	0 x 1	5,178	3.01	0.16	0.24	0.49
	0 x 0	95,109	2.85	-	-	-

462 **Table 2. Analysis of two juvenile mortality rates for different mating types for the Chr28**
 463 **g.4,039,268G>A variant.** “Mating” indicates the genotype for the Chr8 g.4,039,268G>A
 464 variant in allelic dosage (1=heterozygous carrier of the mutant allele; 0= non-carrier) of the
 465 sire and maternal grandsire of the group of individuals considered. For example, “1 x 0” refers
 466 to the progeny of a carrier bull with the daughter of a non-carrier bull. Nb: Number of
 467 observations. SE: Standard error. Difference (%): Difference between the studied mating type
 468 and the control group (i.e., mating type 0 x 0). P-value: Student's t-test. Note the significant
 469 differences between the 1 x 1 and 0 x 0 genotype groups for several periods. Note also that we
 470 found a small but significant +0.47 increase in mortality for the 0-2 day period in matings
 471 between carrier sires and non-carrier sires (1 x 0 vs. 0 x 0) due to the fact that the mutant
 472 allele segregates at a frequency of 2.60% in the maternal granddam population.

473

474 Finally, we studied the effects of the Chr28 g.4,039,268A allele on five performance traits
 475 genetically evaluated each year in the framework of the national polygenic BLUP evaluation.
 476 We found a significant result for only one trait, namely a reduction of one point of muscular
 477 development (MDev) at the age of 210 days in heterozygous carriers versus wild-type

478 individuals ($p=0.04$; Table 3). Note that 1.00 point represents 21% of the genetic standard
479 deviation for this trait (GSD=4.77 points).

480

Trait	Headcount per genotype		B effect (SD)	P- value
	Wild-type	Heterozygous		
Birth weight (in kg)	7,928	488	-0.08 (0.13)	0.54
Ease of calving (score from 1 to 5)	7,926	488	0.00 (0.01)	0.91
MDev at 210 days (score on 100)	4,404	271	-1.00 (0.49)	0.04
SDev at 210 days (score on 100)	4,404	271	0.24 (0.48)	0.61
Weight at 210 days (in kg)	4,146	255	-0.71 (1.14)	0.53

481 **Table 3: Analysis of five performance traits in animals genotyped for Chr28**
482 **g.4,039,268G>A variant.** MDev: Muscular development. SDev: Skeletal development. B
483 effect: effect size. SD: Standard deviation. P-value: Student's t-test.

484

485 Discussion

486 In this article, we set up a powerful approach that led to the identification and characterization
487 of a novel *GNPAT* deep intronic splicing mutation responsible for recessive RCDP in Aubrac
488 cattle. The success of our strategy largely relied on the particular structure of the bovine
489 populations and on the availability of massive amounts of pedigree, genomic and phenotypic
490 information recorded for selection purposes [5].

491 Cattle breeds are genetically small populations created 150 years ago from a limited number
492 of founders, whose genetic variability has been further reduced over the last 50 years by the
493 overuse of influential sires through AI [42,43]. Typically, cattle breeds have effective
494 population sizes (N_e) ranging from 12 to 150, and a minimum number of ancestors
495 contributing to 50% of the breed's gene pool ranging from 5 to 71, as reported in a recent
496 study of 26 cattle breeds reared in France ([https://idele.fr/detail-dossier/varume-resultats-](https://idele.fr/detail-dossier/varume-resultats-2023)
497 [2023](https://idele.fr/detail-dossier/varume-resultats-2023); accessed 2024/03/28). This low genetic variability within breeds contrasts with the very
498 high genetic variability observed at the species level, supported by the discovery in 2019 of
499 84 million SNPs and 2.5 million small insertion-deletions (equivalent to one variant every 31

500 bp) in a collection of 2,703 individuals representing a significant proportion of global cattle
501 population diversity as part of the 1000 Bull Genomes Project [44].

502 As a result, it is possible to capture most of a breed's gene pool by sequencing only its major
503 ancestors (for whom biological material is often available decades after their death in the form
504 of frozen semen straws), and to capture ancient genetic variability by repeating this
505 sequencing effort in numerous independent breeds. This situation explains why, after
506 homozygosity mapping of the RCDP locus in a 1.6-Mb interval on Chr28, we were able to
507 reduce the number of candidate variants from 3,115 to a single one: a deep intronic
508 substitution located in the 11th intron of the *GNPAT* gene (Chr28 g.4039268G>A).

509 Although this type of variant usually does not affect gene expression, in rare situations it can
510 cause severe splicing defects, often resulting in monogenic defects due to partial or complete
511 loss of gene function (e.g. [45,46]). To our knowledge, the only example reported so far in
512 cattle is a SNP in intron 2 of the gene encoding myostatin (*MSTN*), which causes muscle
513 hypertrophy in the Blonde d'Aquitaine breed [47]. As several loss-of-function mutations in
514 the coding part of *MSTN* had been previously described in double-muscled cattle [48,49], the
515 authors used a candidate gene approach: they first sequenced the cDNA of this gene and then
516 its unique exon, after observing an abnormal transcript.

517 Here, since the Chr28 g.4039268G>A substitution was the only remaining candidate variant
518 after a thorough mapping, whole genome sequencing and filtering procedure, we logically
519 assumed that it altered the correct splicing of *GNPAT* and performed complementary *in vitro*,
520 *in vivo*, and *in silico* analyses to validate this hypothesis.

521 As RNA is rapidly degraded at room temperature within a few hours of death, we could not
522 study the expression of *GNPAT* in homozygous mutants. We therefore chose to analyze the
523 splicing of two minigenes (containing intron 11 with either the ancestral or the derived allele

524 of the Chr28 g.4039268G>A substitution and the two flanking exons) in HEK293T
525 transfected cells and, subsequently, of *GNPAT* in blood RNA samples from three
526 heterozygous carriers and three wild-type Aubrac cattle (Fig. 5).

527 Both confirmed that the derived allele was associated with abnormal splicing patterns, likely
528 mediated by an increase in the binding capacity of an SF2/ASF splicing factor encompassing
529 the deep intronic substitution, as predicted by the ESEfinder 3.0 software.

530 The *in vitro* analysis yielded a transcript containing an 86 bp cryptic exon, which was also
531 observed *in vivo*, together with two additional aberrant transcripts corresponding to
532 incomplete splicing of the newly created introns surrounding this cryptic exon.

533 However, while the level of expression of the two minigene constructs were similar *in vitro*,
534 the aberrant transcripts appeared to be less abundant than the correctly spliced ones in the
535 blood of heterozygous animals. We suggest that this difference is due to degradation of the
536 misspliced transcripts containing premature termination codons (PTC) by non-sense-mediated
537 mRNA decay (NMD) *in vivo* but not *in vitro*. In fact, NMD cannot occur in the context of the
538 minigene because the PTC is located in the last exon, which is not the case in the context of
539 the full gene sequence *in vivo*.

540 In any event, translation of transcripts containing all or part of intron 11 would result in the
541 production of proteins lacking the last 21% amino acids of the bovine *GNPAT* protein (Figure
542 5e), and in particular the peroxysomal targeting signal 1, which is essential for sorting the
543 majority of peroxysomal proteins to this organelle [50,51].

544 Therefore, whether due to NMD or to frameshifts and targeting errors, our results suggest that
545 the splicing defects caused by the Chr28 g.4039268G>A deep intronic mutation will
546 ultimately lead to a major reduction in the amount of functional *GNPAT* protein in the
547 peroxysomes of homozygous mutants.

548 The glycerone-phosphate O-acyltransferase (GNPAT), also known as dihydroxyacetone
549 phosphate acyltransferase (DAP-AT, DAPAT and DHAPAT), is an enzyme located
550 exclusively in the peroxisomal membrane that mediates the first step in the synthesis of ether
551 phospholipids including plasmalogen [52].

552 As for other proteins involved in peroxisomal protein import (PEX5 and PEX7) or ether
553 phospholipid synthesis (AGPS and FAR1), mutations in the gene encoding GNPAT have
554 been reported to cause RCDP in humans [15–20].

555 RCDP is a severe developmental disorder caused by defects in plasmalogen synthesis.
556 Patients with RCDP present with skeletal dysplasia including rhizomelic shortening of the
557 limbs, characteristic punctate epiphyseal calcifications, and a typical dysmorphic facial
558 appearance with a broad nasal bridge, epicanthus, high-arched palate, micrognathia, and
559 dysplastic external ears. Clinical features also include congenital cataracts, contractures,
560 seizures, severe growth and psychomotor retardation, and markedly shortened life span
561 [16,53,54]. The degree of plasmalogen deficiency, which depends on the gene and type of
562 mutation involved, determines the severity of the syndrome [55]. For example, erythrocyte
563 plasmalogen levels are almost undetectable in classical severe RCDP, whereas they reached
564 up to 43% of average controls in a study focused on 16 patients with mild RCDP [56]. To our
565 knowledge, no deep intronic mutation in *GNPAT* has been reported in humans. However,
566 based on a literature review, we identified three mutations that, similar to the bovine mutation
567 reported here, are predicted to generate NMD-targeted mRNAs and proteins truncated in the
568 C-terminal region (*GNPAT* c.1428delC, c.1483delG, and c.1575delC causing frameshifts
569 starting at amino acid positions 477, 495, and 525, respectively; [57;58;17]. Plasmalogen
570 levels measured in patients homozygous for any of these three deleterious variants were
571 undetectable or close to zero, suggesting that they do not produce functional GNPAT proteins.
572 Furthermore, neither GNPAT activity nor GNPAT protein was detected in cultured fibroblasts

573 from patients with the *GNPAT*^{c.1428delC/ c.1428delC} and *GNPAT*^{c.1483delG/ c.1483delG} genotypes
574 [57,58]. Here, because of some limitations due to the stillbirth and freezing of the necropsied
575 specimens, we were not able to examine the neuromuscular manifestations or measure
576 plasmalogen levels, which are traditionally evaluated in red blood cells by gas
577 chromatography/mass spectrometry [59]. However, based on imaging and skeletal
578 examination of three calves homozygous for the *GNPAT* deep intronic variant, we observed
579 rhizomelic shortening of the limbs and punctate epiphyseal calcification, which are the
580 hallmarks of the classic severe form of RCDP along with multiple craniofacial malformations.
581 Thanks to the combination of large-scale genotyping and the mining of pedigree and
582 performance records available in the French National Cattle Database, we also documented
583 increased levels of juvenile mortality in the offspring of at-risk versus control mating (with
584 some cases dying at birth and others within the following two weeks), consistent with full
585 penetrance of the mutation in the homozygous state. Taken together, these observations
586 further support our expectation that homozygosity for the bovine Chr28 g.4039268A allele
587 results in severe *GNPAT* and plasmalogen insufficiency.

588 In addition, we would like to point out that, unlike the affected calves reported in this article,
589 the clinical features of *GNPAT*-deficient mice are not entirely consistent with those
590 commonly reported for human RCDP. Mice homozygous for a targeted invalidation of the
591 *Gnpat* gene exhibited a complete lack of plasmalogens, male infertility, defects in eye and
592 central nervous system development, abnormal behavior, and mild skeletal abnormalities
593 consisting of disproportionate dwarfism with shortening of the proximal limbs [60]. *Gnpat*
594 KO mice were viable and while some of them died prematurely (~40% within the first four to
595 six weeks), others, especially females, were long-lived. This difference between humans and
596 mice in the severity of clinical features associated with inactivation of a gene associated with
597 RCDP was also observed for *Pex7* (reviewed in [61]). This finding suggests that calves

598 homozygous for the Chr28 g.4,039,268A allele could be used as a reliable large animal model
599 for RCDP, as an alternative to mouse models, especially to study how impairment of
600 plasmalogen biosynthesis may affect the process of endochondral ossification in this
601 pathology.

602 The efficient identification of heterozygous carriers by genotyping of the GNPAT deep
603 intronic mutation as part of the genomic evaluation, and the mastery of reproductive
604 biotechnologies in livestock breeding make it possible to envisage the production of case and
605 control individuals in experimental farms for further functional analyses and translational
606 research between cattle and humans. Indeed, techniques such as oestrus synchronization,
607 polyovulation, embryo collection, preimplantation diagnosis, embryo freezing and embryo
608 transfer have been successfully used over the past two decades to study developmental
609 processes such as horn ontogenesis in bovine fetuses (e.g., [62–64]).

610 Finally, mining performance records for thousands of genotyped cattle we report a significant
611 reduction of muscular development at the age of 210 days in heterozygous carriers versus
612 wild-type individuals ($p=0.04$; $n=271$ and $4,404$ individuals respectively). The magnitude of
613 this reduction, which represents 21% of the genetic standard deviation for this trait, is similar
614 to that of a QTL with a relatively high effect. This result is consistent with the observations of
615 Dorninger et al. [65], that Gnpat KO mice have altered development and function of the
616 neuromuscular junction, causing reduced muscle strength, and advocates for the fine
617 phenotypic characterization of muscle development and function in humans heterozygous for
618 GNPAT deleterious mutations.

619 **Conclusions**

620 In conclusion, this study highlights the usefulness of large data sets available in cattle for (i)
621 detecting causative mutations beyond the coding regions and (ii) characterizing their

622 phenotypic effects, as exemplified by the report of the first large animal model of RCDP in
623 humans caused by a deep intronic splicing mutation of *GNPAT*.

624

625 **Declarations**

626 **Ethics approval and consent to participate**

627 Experiments reported in this work comply with the ethical guidelines of the French National
628 Research Institute for Agriculture, Food and Environment (INRAE). No permit for
629 experimentation was required by law (European directive 2010/63/UE) since the affected
630 animals were not purposely generated for this study and since all invasive exams and
631 sampling were performed post-mortem on animals that died of natural death. Blood was
632 collected during routine sampling (for annual prophylaxis, paternity testing, or genomic
633 selection purpose) by trained veterinarians and following standard procedures and relevant
634 national guidelines. All the samples and data analyzed in the present study were obtained with
635 the permission of the breeders and of the “OS Race Aubrac” breed organization.

636

637 **Consent for publication**

638 Not applicable

639

640 **Availability of data and materials**

641 The WGS data of the RCDP-affected calves are available at the European Nucleotide Archive
642 (www.ebi.ac.uk/ena) under the study accession no. PRJEB76441.

643

644 **Competing interests**

645 The authors declare that they have no competing interests.

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649

650 **Authors' contributions**

651 A. Boulling and AC conceived and coordinated the project. JC, VP, AC, and JM performed
652 necropsy and pathological examination. A. Barbat and CG analyzed pedigree information. CG
653 supervised sample collection and preparation for SNP array genotyping and whole genome
654 sequencing, and performed PCR and Sanger sequencing. AC analyzed SNP array genotypes.
655 MB and AC analyzed whole-genome sequences. A. Boulling and LBB performed *in vitro*, *in*
656 *silico* and *in vivo* analyses. AC contributed to *in silico* analyses. A. Barbat analyzed juvenile
657 mortality. ST analyzed performance traits. HL, SF, CL, AD, RG and DB contributed
658 reagents/materials/analysis tools. A. Boulling, AC and JC drafted the manuscript. All authors
659 read and approved the final manuscript.

660

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667

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850 **Figures**

851 **Figure 1 Pedigree analysis.**

852 (a) Graph showing the genetic contribution to the case group (n=17) and the ratio
853 "contribution to the cases / contribution to 110,247 controls" for ancestors with a genetic
854 contribution greater than or equal to 1% in each population. (b) Simplified pedigree of 12
855 cases descending from ancestor « E. ». Squares and circles represent males and females
856 respectively. Gray filled symbols correspond to affected calves. *: Animals necropsied. #:
857 Individuals selected for whole genome sequencing.

858

859 **Figure 2 Macroscopic view of Aubrac « Bulldog » calves and characterization of their** 860 **craniofacial dysmorphism.**

861 (a-b) General view and detail of the head of affected calves with facial features reminiscent of
862 those of the French bulldog, hence the name given to this pathology by breeders. (c-d)
863 Longitudinal section of the skull of a case and control calf, respectively. e) Radiograph of the
864 head of an affected calf. (f-g) CT scan images of the head of a case and a control calf,
865 respectively. The black arrowhead points to the anterior fontanelle between the occipital bone
866 and the two frontal bones observed in affected calves. Fn: Frontal bone. In: Incisive bone.
867 Mn: Mandible. Mx: Maxillary bone. Ns: Nasal bone. Oc: Occipital bone. Zy: Zygomatic
868 bone. Scale bars = 10 cm.

869

870 **Figure 3 Imaging and skeletal preparation of the limbs of "Bulldog" and control calves.**

871 (a-b) CT scans of the anterior (a) and posterior (b) limbs of the case. (c-d) Radiographs of the
872 limbs of the same RCDP affected individual. (e-f) Radiographs of the anterior (e) and

873 posterior (**f**) limbs of a control individual. **g**) Skeletal preparation of the left hind limb of case
874 and control calves. **h**) Detail of the humerus shown in (**g**). Note the presence of multiple
875 punctate calcifications where the epiphyses should be found and the absence of the femoral
876 head (visible in (**h**)) and the tuberosity of the calcaneus (indicated by a white arrowhead in (**d**)
877 and (**f**)). Cr: Cranial orientation. Scale bars = 10 cm.

878

879 **Figure 4 Mapping and identification of a candidate causative mutation in *GNPAT* intron**
880 **11.**

881 (**a**) Manhattan plot of homozygosity mapping results with phased and imputed Illumina
882 BovineSNP50 array genotypes from 21 RDCP-affected and 1548 control individuals. The
883 0.05 significance threshold was set at $-\log P=6.20$ after Bonferroni correction of Fisher's exact
884 test p-values. (**b**) Details of the genes located within the mapping interval. The dashed line
885 indicates that the Homo C1orf198 gene encompasses the left border of the interval. (**c**) Details
886 of the localization of the g.4,039,268G>A candidate variant in the 11th intron of *GNPAT*. (**d**)
887 Integrative Genomic Viewer screenshot showing homozygosity for this substitution in the
888 whole genome sequence of an affected calf compared to a control.

889

890 **Figure 5 *In vitro*, *in silico* and *in vivo* analysis of the effect of the g.4,039,268G>A**
891 **substitution on the *GNPAT* splicing.**

892 (**a**) Minigene analysis: Illustration of the pcDNA3.1-*GNPAT* minigenes carrying the derived
893 or ancestral alleles in *GNPAT* intron 11 (upper and lower left panels, respectively) and results
894 of the RT-PCR and gel electrophoresis after transfection of HEK293T cells with the
895 pcDNA3.1-*GNPAT*_A (A) and pcDNA3.1-*GNPAT*_G (G) minigenes (right panel). cDNAs
896 were amplified with primers targeting the pcDNA3.1 5'UTR and 3'UTR regions (green

897 arrows). Two major PCR products were detected (labeled 1 and 2). MW: Molecular weight.
898 **(b)** Splicing patterns associated with the two minigenes based on Sanger sequencing of the
899 amplicons shown in **(a)**. cE: Cryptic exon. **(c)** Sequence details of the cryptic exon: The
900 g.4,039,268G>A substitution increases the score for a predicted SF2/ASF binding site located
901 in its 5' region according to the ESEfinder 3.0 software. **(d)** *In vivo* analysis of *GNPAT*
902 transcripts. Left panel, representative subset of the results obtained after gel electrophoresis
903 following RT-PCR on total blood RNA extracted from wild-type (WT) and heterozygous
904 (HT) Aubrac cattle using primers targeting *GNPAT* exons 11 and 12. Four observed PCR
905 products are numbered and their structures are shown (see text for details). MW: Molecular
906 weight. **(e)** Consequences of the splicing patterns shown in **(d)** on the primary structure of the
907 *GNPAT* protein. Normal amino acids (AAs) are shown in green, novel AAs are shaded. The
908 acyltransferase motif (AAs 162 to 167) and the peroxysomal targeting signal 1 (PTS1, AAs
909 678 to 680; (18)) are marked with an asterisk.

910

911 **Additional files**

912 **Additional file 1: Table S1**

913 Format: xlsx

914 Title: Details of the whole genome sequences used as controls in this study.

915 Description: See the following URLs for more information on the biosample and bioproject

916 IDs: <https://www.ncbi.nlm.nih.gov/biosample/> and <https://www.ncbi.nlm.nih.gov/bioproject/>.

917 Nb_Ind_Breed: Number of individuals per breed.

918 **Additional file 2: Figure S1**

919 Format: pdf

920 Title: Radiographs of an affected calf.

921 Description: Cr: cranial orientation. Scale bar = 10 cm.

922 **Additional file 3: Table S2**

923 Format: xlsx

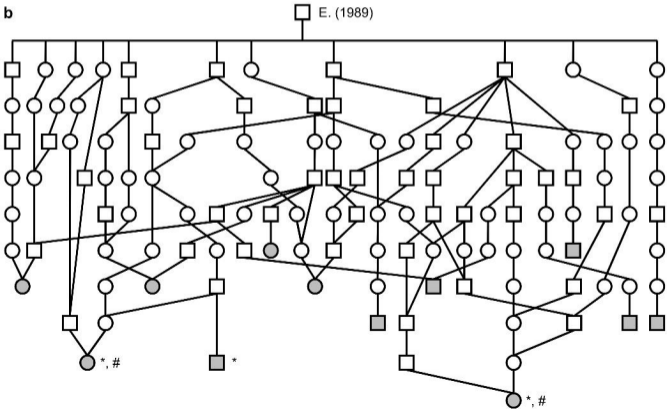
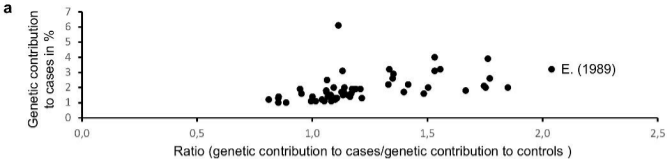
924 Title: List of homozygous positional candidate variants found in the genomes of two RDCP-

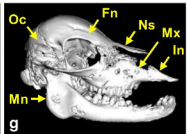
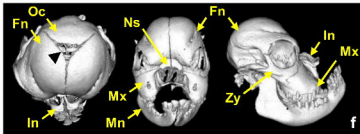
925 affected calves

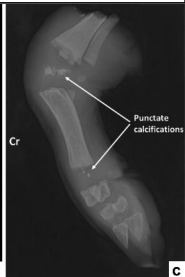
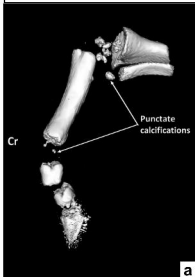
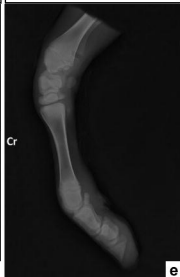
926 Description: Chr: Chromosome. "Present_in_controls" indicates whether the variant was

927 observed in at least one of the 1,867 genomes used as controls (see Additional file 1: Table

928 S1).



Cases**Control**

Cases**Control****Case/Control**